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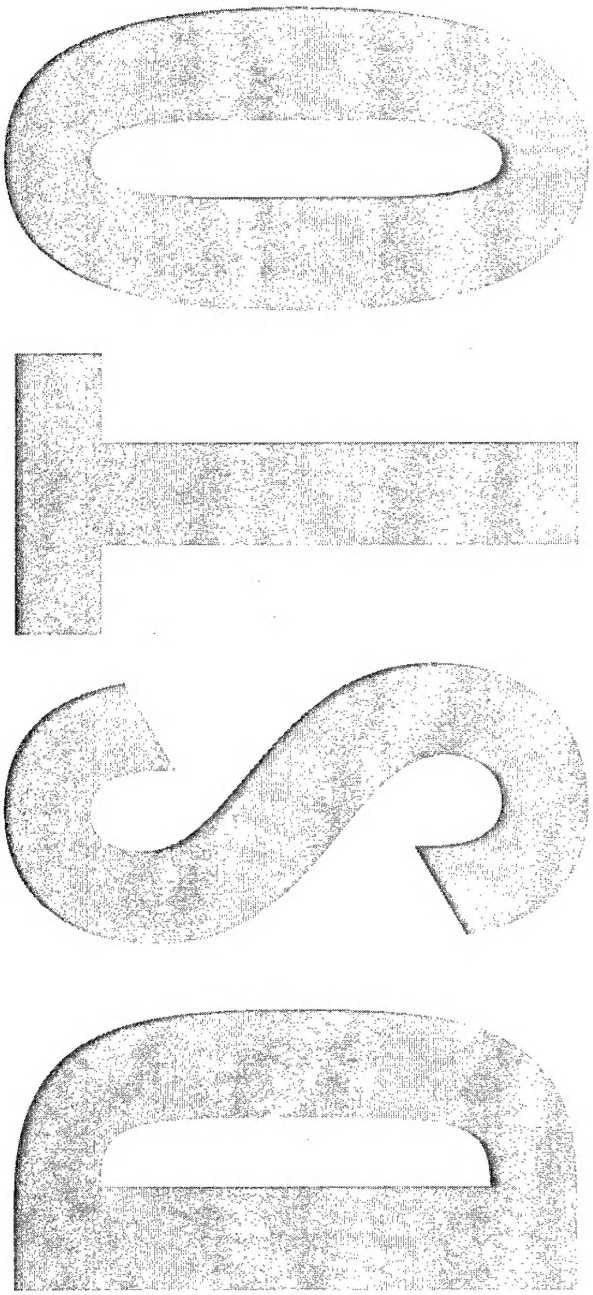

Methods for the Production of Antibodies Against Potential Biological Warfare Agents

Malcolm R Alderton and
Penelope J Gauci

DSTO-TR-1619

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CBRN Defence Centre
Platforms Sciences Laboratory

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ABSTRACT

This report details the procedures used to produce mouse monoclonal and polyclonal antibodies with affinity for the etiological agent of the plague (Yersinia pestis) and the plant toxin ricin. It also details the inability to produce monoclonal or polyclonal anti-Coxiella burnetii antibodies in mice using the CSL Q-fever vaccine as a Coxiella antigen.

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Executive Summary

Difficulties in obtaining reagents with high affinity for many microbial pathogens and toxins that are potential biological warfare (BW) agents from commercial companies, Universities and research institutes resulted in DSTO initiating a program to produce monoclonal and polyclonal antibodies with affinities for the plant toxin ricin and the causative agents of plague and Q fever. They also led to anti-anthrax antibodies being produced under contract by the South Australian Institute for Medical and Veterinary Science (SAIMVS) and the Department of Biochemistry at the University of Adelaide. Antibodies with high affinities for the microbial pathogens were required for the development of ion channel switch (ICS) biosensors in a Defense Advanced Research Project Agency (DARPA) funded collaborative research project with the Australian Membrane and Biotechnology Research Institute (AMBRI). High affinity antibodies were also required for the development of traditional immunoassays, *eg* ELISA, for the detection of the potential BW agents identified above.

Antigen binding fragments (Fab) of the monoclonal antibodies with affinity for the plant toxin ricin, and polyclonal antibodies with affinity for ricin, the F1 antigen of *Y. pestis* and *B. anthracis* (causative agent of anthrax), were generated by proteolytic digestion with pepsin and tested in ICS biosensors for the detection of these agents. Although the anti-*Yersinia pestis* (causative agent of plague) monoclonal antibodies had affinity for the pathogen, difficulties in digesting the antibodies with chymopapain and pepsin meant the antigen binding fragments (Fab) required for use in the ion channel switch (ICS) biosensor were never produced. However, they were used effectively in ELISA.

The generation of high affinity reagents that can be used to develop novel immunoassays, including ICS biosensors, for the rapid detection of potential BW agents will provide the Australia Defence Force (ADF) with an early warning system for these agents.

Authors

Malcolm Alderton
CBRN Defence Centre

Malcolm Alderton graduated from Monash University with a B Sc (Hons) in 1980 and a PhD (Immunology) in 1984. In 1994 he graduated with a Grad Dip Ed from RMIT and in 2000 he graduated with a Grad Cert Man from Flinders University. In his first postdoctoral position with Biotechnology Australia he worked on the coccidiosis project at CSIRO Animal Health (Parkville). He moved to RMIT University in 1988 and worked as a Research Scientist and Lecturer before joining DSTO in 1995 as a Research Scientist. In January 1997 he was promoted to Senior Research Scientist and in 1998 became a Principal Investigator in a collaboration with the Australian Membrane and Biotechnology Research Institute (DSTO) Pty Ltd to develop ion channel switch (ICS) biosensors for defence applications. In July 2000 he became a Task Manager, and in March 2001 he started a 12 month sabbatical with the CSIRO Health Sciences and Nutrition Division. Between 2001 and 2004 he managed the Novel Countermeasures Against BW Agents Task, and currently manages the CBRN DC Toxin Project.

Penelope J. Gauci
CBRN Defence Centre

Penny Gauci graduated from the University of Melbourne with a B Sc in 1992. She joined the Department of Applied Biology and Biotechnology, RMIT University as a Technical Assistant in 1992, before being promoted to Technical Officer in 1993. Her work at RMIT included production and purification of polyclonal and monoclonal antibodies, antigen purification, chromatography, vaccine production and animal trials for vaccine efficacy testing. She joined AMRL in 1998 and worked predominantly on the production of antibodies for use in ion channel switch (ICS) biosensors. She joined the DNA vaccines program in 2001, and began work on the production of DNA vaccines for Burkholderia pseudomallei.

Contents

1. Introduction

Biological weapons have been used sporadically for centuries. Throwing dead people, horses or livestock into wells, lakes and rivers used for drinking water and catapulting bodies of plague victims into walled cities are examples of how biological agents have been used in the past (1). In the twentieth century a number of countries established programs for the production and dissemination of biological weapons. Fortunately, the list of microbial pathogens and toxins that can be used as BW agents is quite short. A North Atlantic Treaty Organisation handbook on biological warfare lists 39 bacteria, viruses and toxins as potential biological weapons (2). A number of the agents on this list can be eliminated as effective biological weapons because of difficulties associated with their culture, storage and dissemination (3). However, the etiological agents of anthrax, plague and Q-fever – *Bacillus anthracis*, *Yersinia pestis* and *Coxiella burnetii* respectively – are examples of bacterial pathogens on the list likely to cause high numbers of casualties if used as a biological weapon.

The most recent attacks in the United States using letters containing *Bacillus anthracis* spores highlight the current deficiency in effective human vaccines for microbial pathogens and toxins that could be used as biological warfare agents. This deficiency is further highlighted by the fact that in a list of potential BW agents (4) there were only vaccines for approximately half the agents. In recent years there has been a concerted effort by military researchers to develop rapid detection systems, e.g.: immunoassays/biosensors, and antibody-based therapy or prophylaxis for these agents (4, 5). These detection systems are only as good as the quality of antibody, i.e. the strength of binding (avidity) between the antibody and the antigen. Unfortunately, the production of neutralizing antibodies with high affinity for many microbial pathogens and toxins that are potential biological warfare agents, for example *Bacillus anthracis* spores and the plant toxin ricin, is generally difficult and time consuming. Until recently, traditional methods of antibody production involving animal immunization, including monoclonal antibody production, were the preferred way of producing antibodies.

This report describes the production of polyclonal and monoclonal antibodies to potential BW agents - ricin, *Y. pestis* and *C. burnetii* - at DSTO for use in ion channel switch biosensors and immunoassays. Polyclonal and monoclonal antibodies to *B. anthracis* spores were produced by the South Australian Institute for Medical and Veterinary Science (SAIMVS) and the Department of Biochemistry, University of Adelaide under contract, but the procedures used are not described in this report.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

Eight to ten week-old female Balb\c mice were obtained from Monash Animal Services.

2.1.2 Reagents for animal inoculation

Pristane (2,6,10,14-tetramethylpentadecane) and Freund's incomplete adjuvant (FIA) were purchased from Sigma-Aldrich. Needles and syringes were purchased from Terumo Medical Corporation.

2.1.3 Antigens used in mouse inoculations

Inactivated vaccine organisms or bacterial antigens were used in animal inoculations for the generation of antibodies to *Y. pestis* and *C. burnetii*. The human plague vaccine containing heat inactivated *Y. pestis* organisms and the human vaccine containing *C. burnetii* antigens were obtained from CSL limited.

These microbial antigens were washed thrice in PBS and resuspended to the required concentration in the same buffer prior to use in animal inoculation.

Ricin, ricin peptide A and ricin peptide B used for inoculations of mice were purchased from Sigma - Aldrich Pty Ltd. Ricin, ricin peptides and *Coxiella burnetii* antigens were emulsified in Freund's Incomplete Adjuvant (FIA) before being used to inoculate mice. Washed and heat inactivated *Y. pestis* organisms were used without emulsification in FIA. The antigen complexity of bacterial cells ensures a strong immune response without the need for an adjuvant.

2.1.4 Reagents and equipment for monoclonal antibody production

The Sp/2 myeloma cell line was purchased from CSL limited. Polyethylene glycol 1500 was purchased from Sigma-Aldrich. All cell culture medium ingredients - Dulbecco's modification of Eagles medium (DMEM), sodium bicarbonate, Hepes, L-glutamine, penicillin/streptomycin, bovine calf serum, 50 x HAT (hypoxanthine, aminopterin and thymidine) and 50 x HT - were obtained from Trace Biosciences Pty Ltd. Sterilised Milli-Q water was prepared in the Laboratory. Flat-bottomed 96-well microtitre plates used to culture hybridomas were obtained from Nalge Nunc International.

2.1.5 Reagents and equipment for testing polyclonal ascites and screening hybridoma supernatants

The 0.05M carbonate-bicarbonate buffer, pH 9.6 used to adsorb antigen to the microtitre plates was prepared in the laboratory using sodium carbonate and sodium bicarbonate obtained from Ajax Chemicals.

The flat-bottomed 96-well Maxisorp plates used in ELISAs to test polyclonal ascites and screen hybridoma supernatants for antibodies to the target antigens were obtained from Nalge Nunc International.

PBS/Tween tablets, a goat anti-mouse alkaline phosphatase-labelled conjugate and the p-phenylphosphate substrate were all purchased from Sigma-Aldrich.

2.2 Methods

2.2.1 Inoculation procedures for polyclonal ascites production

2.2.1.1 Inoculation of mice with Yersinia pestis

After the initial pristane injection, the female Balb\c mice received a series of intraperitoneal *(ip)* inoculations containing washed *Y. pestis* organisms. The initial antigen inoculation contained 60 million organisms in PBS. The additional *ip* antigen injections contained between 5 and 10 million organisms. These injections were given at 2-weekly intervals.

2.2.1.2 Inoculation of mice with Coxiella burnetii

One week after the initial pristane injection, the mice received a series of *ip* injections containing between 2 and 3μg of washed *C. burnetii* antigens in FIA. These injections were given at 2-weekly intervals.

2.2.1.3 Inoculation of mice with ricin

All mice used to produce polyclonal anti-ricin antibodies were between 8 and 10 weeks old when they received a 0.25ml intraperitoneal *(ip)* injection of pristane to start the inoculation regime. One to four weeks after the pristane injection, the mice received the first of a series of injections of ricin–, ricin peptide A–, ricin peptide B–FIA emulsions or combinations of these reagents. Mice inoculated with ricin received between 0.5 and 1.0 μg of the toxin in each injection, whereas, mice inoculated with ricin peptide A or ricin peptide B received between 1.0 and 3.0 μg, and 3.0 and 5.0 μg of the peptide in each injection, respectively. All mice inoculated with ricin for the production of polyclonal antibodies were hyperimmunised, receiving between six and eight injections over 10 to 16 weeks. As with the other antigen inoculation regimes described above, all ricin injections were given *ip* except the last, which was given subcutaneously.

2.2.2 Polyclonal ascites production

Between 10 and 14 days after the final antigen injection in all the inoculation procedures described above the mouse received an *ip* injection of between 10 and 20 million Sp/2 myeloma cells.

The ascitic fluid, which contained the antibody, was drained from the peritoneal cavity of the animals using a 20-gauge needle 10 and 14 days after the injection containing the myeloma cells. The fluid was centrifuged at 500x*g* for 10min to remove any cellular contaminants and stored at -20 C.

The IgG fraction that contains the higher affinity antibodies was isolated from the ascites using a Protein G column. The potency of the IgG fraction was determined by testing serial dilutions in an ELISA. The serial dilutions were generally doubling dilutions that started at 1 in 200 or 1 in 250 and finished at 1 in 28, 600 or 1 in 64, 000.

2.2.3 Inoculation procedures for monoclonal antibody production

A flow diagram detailing the steps used in production of monoclonal antibodies is provided in Figure 1. This should enable the reader to understand the basic procedure from mouse immunization to the production and assessment of large volumes of monoclonal antibodies, and where the methods described below fit in this procedure.

Similar inoculation regimens to that used for the production of anti- *Y. pestis*, -*C. burnetii* and -ricin polyclonal antibodies were used for the production of anti -*Y. pestis*, -*C. burnetii* and -ricin monoclonal antibodies, except none of the mice received a pristane injection.

2.2.4 Monoclonal antibody fusions

2.2.4.1 Feeder cells

In order to provide fused cells with the best environment for growth, they were cultured initially in the presence of feeder cells. These feeder cells, a collection of lymphocytes and phagocytic cells, were collected aseptically from the peritoneal cavity of Balb\c mice. This involved killing a mouse and sterilising the fur by immersing it in 70% alcohol for 2-3 minutes. After removing the excess alcohol with Kimwipes, an incision was made in the fur from the ribs to the base of the abdominal cavity. Care was taken not to penetrate the skin covering the cavity. By carefully pulling the edges of the incision, the fur was peeled off the abdominal cavity. Five to seven millilitres of serum-free DMEM was then injected into the peritoneal cavity using a 10 ml syringe and a 26-gauge needle. After gently, and aseptically, massaging the abdominal cavity of the mouse the medium was removed using a 10 ml syringe and an 18-gauge needle. Extreme care was taken not to perforate the intestines during the extraction. The number of feeder cells obtained was determined using a hemacytometer. The cells were then pelleted and resuspended in DMEM containing 10% fetal calf serum and 0.1% penicillin/streptomycin (10% FCS-DMEM) at a concentration between 50 million and 100 million cells/ml. One hundred microlitres of the

feeder cell suspension was then added to each well of four 96-well microtitre plates, and the plates incubated overnight at 37°C in a atmosphere containing 5% CO_2.

2.2.4.2 Monoclonal antibody fusions

B-lymphocytes from immunized mice were fused with a myeloma cell line (SP2/0) according to standard protocols (6). Two mouse spleens were used in most fusions, and the resulting cells fused with myeloma cells in a ratio of 5:1 during the drop-wise addition of 0.8 ml of polyethylene glycol (PEG) 1500 over 2 min at 37° C. After the cells were washed and pelleted they were resuspended in 40 ml of 10% FCS-DMEM containing 2% HAT (complete medium). One hundred microlitres (100 µl) of this cell suspension was added to each well in the culture plates containing the feeder cells. The plates were incubated at 37°C in an atmosphere containing 5% CO_2 for 3 days before 120 µl of medium was removed and replaced with 120 µl of complete medium. Thereafter, medium was removed and replenished as required, *ie* when the supernatant became acidic and turned yellow.

2.2.5 Screening of hybridoma supernatants

Supernatants of wells containing hybridomas were screened for antibodies specific for the antigen used to inoculate the mice by ELISA. Immunoblots were used as an alternative screening method.

2.2.5.1 ELISA

Antigens in 100µl of 0.05M carbonate-bicarbonate buffer (pH 9.6) were adsorbed to the wells of a Nunc Maxisorp 96-well microtitre plate in an overnight incubation.

Y. pestis antigens used in ELISA to screen hybridoma supernatants were obtained by sonicating washed vaccine organisms (100 million/ml, on ice) for 6x60 seconds, setting 3 (50%) using a Branson Sonifier 250. The resultant mixture was centrifuged at 500xg to remove any particulate matter, and dialysed against 0.1M carbonate/bicarbonate buffer, pH 9.6. After a 24-hour incubation the volume of the antigen solution was increased to 5 times the vaccine volume using the same buffer. One hundred microlitres of this solution was added to the wells of a Nunc Maxisorp 96-well microtitre plate, and the antigen adsorbed to the well during an overnight incubation at room temperature. One hundred microlitre volumes of carbonate/bicarbonate buffer containing 0.1 - 0.25 µg of ricin, ricin peptides and washed *C. burnetti* antigens were used to coat the wells of ELISA plates with these antigens.

Following the antigen-adsorption to the plate, the reagents were applied in the following order:

1. Blocking agent - 3% skim milk in PBS/tween (PBS/T)
2. Hybridoma supernatants
3. Goat anti-mouse alkaline phosphatase
4. Substrate - p-nitrophenyl phosphate.

Three washes with PBST were performed after antigen adsorption and between the removal of one reagent and the addition of the next, except for the removal of the alkaline phosphatase conjugate and the addition of the substrate, when there were 4 washes: three in PBST and one in distilled water. After the addition of each reagent, the plate was incubated at room temperature for one hour. All volumes used were 100ul/well, except for the blocking agent when 300ul/well was used. The supernatants were used undiluted, and the conjugate at a dilution of 1 in 2000. Absorbance readings at three times the negative control (PBST was used instead of a hybridoma supernatant) when the plates were read at 405 nm were considered to contain antibodies to the target antigen.

2.2.6 Cloning by limiting dilution

A limiting dilution protocol was used to generate clones of each hybridoma producing monoclonal antibodies. In brief, cells suspensions containing 30, 10 and 3 hybrids/ml were prepared, and dispensed (100µl/well) into individual microtitre plates. Hybridomas that grew in the 0.3 cells/well plate were likely to be clonal, i.e. been derived from a single hybridoma cell and produce one specific antibody, and were used in the repeat of the same procedure. Hybridomas in the 0.3 cell/well plate of the second limiting dilutions were then used in a third and final round of dilutions. Antibodies produced by hybridomas in the 0.3 cells/well plate of the third limiting dilution were considered monoclonal, and were used to produce monoclonal ascites.

2.2.7 Monoclonal ascites production

Eight to ten week-old female Balb\c mice received an *ip* injection of between 10 and 20 million antibody-producing hybridoma cells. The ascitic fluid was removed and purified as described above in section 2.2.2.

3. Results and Discussion

The inoculation of mice with ricin was performed using sub lethal doses of the toxin, or the A and B peptides alone. Most mice used in the study received six or more antigen injections and were considered hyperimmune when their spleen was removed for hybridoma production or the animal received an intraperitoneal injection of myeloma cells for the production of polyclonal antibodies.

3.1 Polyclonal ascites

The administration of the SP/2 myeloma cells into the peritoneal cavity of the mouse following the series of antigen injections, produced up to 10ml of ascitic fluid that generally contained high levels of antibodies to the target antigen.

Anti-*Y. pestis*, -*C. burnetii* and ricin polyclonal ascites were produced using the procedures described above. IgG fractions of anti-*Y. pestis* and anti-ricin ascites exhibited high titres for the antigen when tested by ELISA. The anti-*C. burnetii* ascites contained few antibodies that reacted with the target antigen. Investigation of this unexpected outcome revealed that low-level *Coxiella* infections in mice were endemic in Australian animal houses, making the generation of moderate to high affinity antibodies to *C. burnetii* in mice almost impossible. Mice with these infections will either become tolerant to the antigens, or have low-levels of circulating antibodies that, with the assistance of phagocytic cells, will quickly eliminate the antigens without the need for the vigorous response required to produce high affinity antibodies.

3.2 Monoclonal antibodies

Multiple hybridoma fusions produced monoclonal antibodies with affinities for *Y. pestis* and ricin. No hybridomas producing anti-*C. burnetii* monoclonal antibodies were generated in these endeavours. This was no surprise given the information on *Coxiella* infections of mice provided above. All monoclonal antibodies produced against Y. *pestis* were directed against the surface F1 antigen, which is not surprising given the ubiquity and immunodominant nature of this capsular antigen. Screening ELISA using ricin peptide A, ricin peptide B and the whole molecule as the antigen identified monoclonal antibodies with affinities for all these antigens were produced in these experiments.

After the production of the antibodies described in this report, the Australian Animal Experimentation Ethics Committee (AEEC) outlawed the practice of using mice (or any other animals) for the production of antibody ascites. This proclamation eliminated the most efficient method of producing highly concentrated preparations of polyclonal and monoclonal antibodies. As yet no alternative method has been devised to replace the polyclonal ascites procedure, but scientific companies have devised a variety of methods and equipment to compensate for the inability to produce concentrated monoclonal antibody preparations in animals. Many of the alternatives to monoclonal ascites are inefficient and expensive. In an evaluation of the CELLine system DSTO scientists were unable to achieve the levels of production (1-4mg/ml) claimed by the manufacturer. In an evaluation of the efficiency of the OptiCell system (BioCrystal, Ohio, USA), that is currently underway, DSTO is hopeful of identifying a simple and inexpensive protocol that will generate a concentrated monoclonal antibody preparation.

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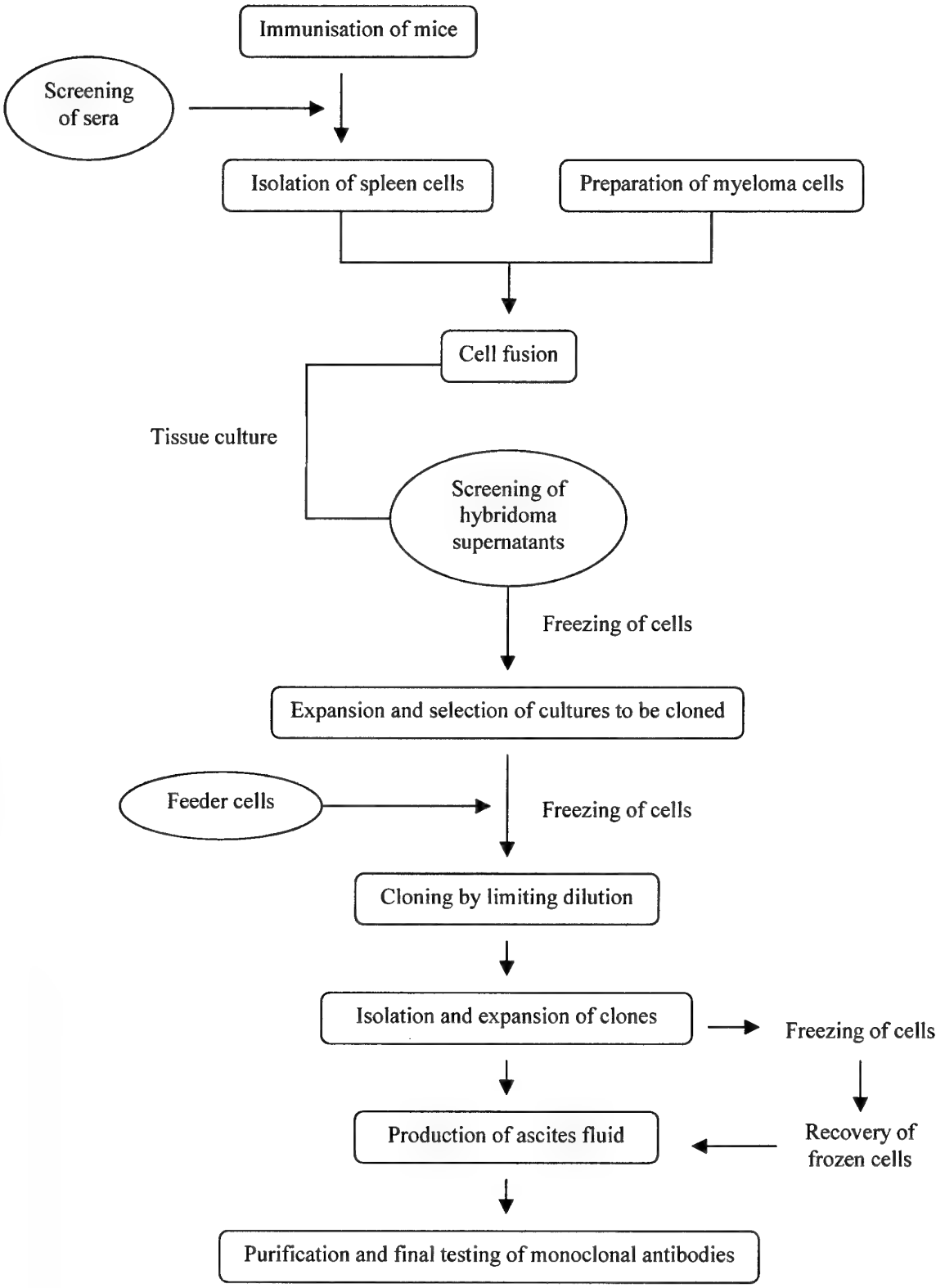

Figure 1. Diagrammatic representation of monoclonal antibody production

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Malcolm R Alderton and Penelope J Gauci

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19. ABSTRACT

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